Burkholderia lata BL02 Galactolipase as an Important Biocatalyst for Plant Biomass Deconstruction and Sugar Ester Synthesis

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Article

Burkholderia Lata BL02 Galactolipase as an Important Biocatalyst for Plant Biomass Deconstruction and Sugar Ester Synthesis

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Abstract

The transition toward sustainable biorefinery processes requires efficient strategies for lignocellulosic biomass deconstruction and valorization. In this study, an integrated enzymatic system combining fungal holocellulases and laccases with a bacterial galactolipase was developed and evaluated. The consortium, composed of *Trametes hirsuta* GMA-01, *Mycothermus thermophilus* CBS 619.91, and *Burkholderia lata* BL02, was produced using agro-industrial substrates and applied to the hydrolysis of different lignocellulosic biomasses. The incorporation of galactolipase activity enhanced saccharification yields for leaf-derived substrates, reaching up to 292.0 mg/g for spinach leaves and 236.0 mg/g for corn straw, compared to fungal systems alone. This effect is associated with the selective hydrolysis of membrane-associated galactolipids, improving substrate accessibility to holocellulolytic enzymes. Proteomic analysis and structural modeling identified the BL02 enzyme as a versatile ester hydrolase with features compatible with accommodating bulky polar substrates. In addition, the enzyme catalyzed the synthesis of sugar fatty acid esters with conversion yields above 50% for glucose and xylose in binary solvent systems. These findings support the role of galactolipases as accessory enzymes and highlight their potential application in integrated and sustainable biorefinery processes.

Keywords: lignocellulosic biomass; enzymatic saccharification; lipase; enzyme cocktail; biorefinery; biocatalysis; biomass valorization

1. Introduction

The transition toward a sustainable bio-based economy has become a central priority in response to the environmental and economic limitations associated with fossil fuel dependence. The continuous growth of the global population and the increasing demand for energy, fuels, and chemical products have intensified the need for alternative production routes that reduce greenhouse gas emissions and promote the efficient use of renewable resources, yet current bioconversion technologies still struggle to achieve industrial-scale efficiency due to structural and biochemical limitations inherent to lignocellulosic substrates [1,2].

In this context, lignocellulosic biomass (LCB) has emerged as one of the most promising renewable carbon sources, enabling the development of integrated biorefinery systems. However, the economic viability of these processes is often limited by the intrinsic recalcitrance of plant cell walls, where cellulose microfibrils are embedded in a complex matrix of hemicellulose and lignin [3,4]. Traditionally, industrial enzymatic cocktails have focused on the "workhorse" enzymes,

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cellulases and hemicellulases. Nevertheless, recent evidence suggests that the efficiency of these consortia is frequently hampered by biophysical barriers, such as membrane-associated lipids and cuticular waxes, which shield the polysaccharide matrix from aqueous enzymatic attack, representing a frequently overlooked bottleneck in lignocellulosic bioconversion processes [5,6].

From a biochemical standpoint, lipolytic enzymes belong predominantly to the α/β-hydrolase fold superfamily. A critical point of differentiation in this group is the structural mechanism of substrate recognition. "True" lipases (EC 3.1.1.3) are characterized by the presence of a structural "lid", a polypeptide loop covering the active site that requires interfacial activation to shift and expose the catalytic triad to hydrophobic substrates [7]. In contrast, esterases (EC 3.1.1.1) and certain atypical lipases lack this restrictive lid, allowing them to act on more polar or water-soluble esters without the need for an interface [8,9].

This structural distinction directly influences substrate specificity and catalytic behavior, particularly in the processing of complex or amphipathic lipids. For instance, cutinases (EC 3.1.1.74), often of fungal origin, exhibit intermediate features, possessing an active site that is much more accessible and can accommodate both soluble esters and insoluble polymers like cutin [10]. This "open" architecture is a recurring theme in enzymes that exhibit significant galactolipase activity (EC 3.1.1.26), which must accommodate the polar head groups of monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG).

Galactolipids are the most abundant lipids in the biosphere, yet their enzymatic deconstruction remains a secondary focus in most biorefinery models [11,12]. While galactolipase activity was historically associated with plant tissues and mammalian pancreatic proteins, microbial systems have recently emerged as superior sources of these biocatalysts, yet their functional integration into lignocellulosic enzyme systems remains poorly understood.

Fungal lipases from *Fusarium solani* and *Talaromyces thermophilus* have demonstrated high catalytic efficiency toward galactolipids, with activities that often rival or surpass those of specialized pancreatic enzymes [13–15]. These enzymes are increasingly recognized for their ability to hydrolyze membrane lipids in complex matrices, such as microalgae and leafy biomass, significantly enhancing the release of fermentable sugars and fatty acids [16]. Furthermore, our research group has identified that the bacterium *Burkholderia lata* BL02 produces a highly robust extracellular lipase/galactolipase. Unlike conventional lipases, this enzyme demonstrates significant activity toward plant-derived galactolipids, suggesting a potential role as an important accessory factor that "clears the path" for lignocellulolytic machinery [17,18].

This study aims to develop and evaluate an optimized enzymatic consortium combining laccase from the basidiomycete *Trametes hirsuta* GMA-01, a holocellulolytic secretome from the thermophilic ascomycete *Mycothermus thermophilus* CBS 619.91, and a lipase/galactolipase from the bacterium *Burkholderia lata* BL02. We investigate the synergistic interactions among these enzymes to enhance the deconstruction of lignocellulosic biomass and enable the subsequent synthesis of high-value sugar esters.

2. Results and Discussion

2.1. Microbial Growth Kinetics and Thermal Optimization for Co-Culture

The secretome of a microbial consortium is heavily influenced by the metabolic compatibility of its members. In this study, we combined the mesophilic white-rot Basidiomycete *Trametes hirsuta* GMA-01 with the thermophilic Ascomycete *Mycothermus thermophilus* CBS 619.91 to obtain a broad spectrum of ligninolytic and holocellulolytic enzymes. A critical prerequisite for successful fungal co-cultivation is identifying a thermal regime that supports the robust development of both species, as their ecological niches (mesophilic vs. thermophilic) are inherently distinct, which directly impacts enzyme secretion profiles and the feasibility of synergistic consortia formation.

As shown in Table 1, the growth kinetics varied significantly across the tested temperatures. *M. thermophilus* exhibited its highest mycelial expansion rates at 35 °C (12.05 ± 2.16 cm2/day) and 40 °C

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(14.80 ± 1.91 cm2/day), with a significant reduction in growth at 30 °C (7.60 ± 1.80 cm2/day, $p < 0.01$). Conversely, *T. hirsuta* maintained similar growth rates at 30 °C and 35 °C (approximately 3.1 cm²/day), followed by a decline at 40 °C (2.30 ± 0.26 cm²/day), indicating thermal stress near its upper physiological limit.

This behavior reflects the ecological adaptation of each species, where thermophilic fungi sustain higher metabolic activity at elevated temperatures, while mesophilic fungi perform optimally under moderate conditions. Based on these results, 35 °C was selected as a compromise temperature for co-culture experiments, as it allows the development of both organisms while maintaining conditions favorable for enzymatic production. These differences in growth kinetics are expected to directly influence secretome composition and enzymatic synergy within the consortium.

Table 1. Mycelial growth rates of *Mycothermus thermophilus* CBS 619.91 and *Trametes hirsuta* GMA-01 at different temperatures (cm²/day).

Fungus	Temperature (ºC)		
	30	35	40
M. thermophilus CBS 619.91	7.60 ± 1.80[a]	12.05 ± 2.16[b]	14.80 ± 1.91[b]
T. hirsuta GMA-01	3.21 ± 0.60[a]	3.06 ± 0.29[a]	2.30 ± 0.26[b]

∗ Mean ± SD (n = 3); Different letters indicate significant differences within the same row ($p < 0.01$).

2.2. Ecological Interactions and Competitive Fitness in Co-Culture

Beyond thermal requirements, the success of a microbial consortium depends on the ecological compatibility of its members. The dual-culture confrontation assays revealed no visible antagonistic interactions between *M. thermophilus* and *T. hirsuta*, as no inhibition zones or pigment diffusion were observed at any tested temperature (Figure 1). This absence of antagonism suggests that both species can coexist under shared cultivation conditions, allowing simultaneous enzyme production without apparent suppression by secondary metabolites.

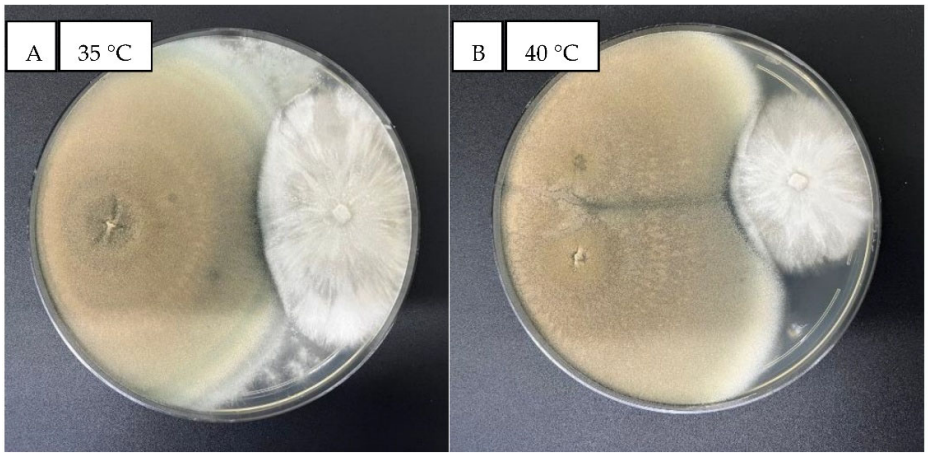

Figure 1. Dual culture confrontation assay between *Mycothermus thermophilus* CBS 619.91 and *Trametes hirsuta* GMA-01 on PDA plates (150 mm diameter) at (A) 35 °C and (B) 40 °C. *M. thermophilus* is positioned on the left side of the plate and *T. hirsuta* on the right.

However, a clear difference in competitive fitness for spatial colonization was observed. As shown in Figure 1, *M. thermophilus* exhibited a dominant growth pattern, progressively overgrowing *T. hirsuta* at both 35 °C and 40 °C. This behavior is consistent with its significantly higher growth rate (Table 1) and reflects its thermophilic adaptation. At 40 °C (Figure 1B), this dominance was further enhanced due to the thermal stress imposed on the mesophilic *T. hirsuta*, resulting in near-complete surface colonization by *M. thermophilus*.

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These findings indicate that, although no antagonistic interactions were detected, *M. thermophilus* displays competitive dominance in substrate colonization. This imbalance may affect the relative contribution of each species to the overall secretome, potentially impacting enzymatic diversity and efficiency. Therefore, the development of an optimized consortium requires strategies to balance this interaction, such as staggered inoculation or adjusted inoculum ratios, to ensure the effective contribution of the ligninolytic system of *T. hirsuta*.

2.3. Evaluation of Enzymatic Secretomes Across Different Agro-Industrial Residues

The enzymatic profiles varied significantly depending on the lignocellulosic substrate used as an inducer. In this study, the inductive potential of corn cob (CC), corn straw (CS), and sugarcane bagasse (SCB) was evaluated to stimulate the secretome of *M. thermophilus* and *T. hirsuta*, both in isolated and co-culture systems. Due to differences in cellulose, hemicellulose, and lignin composition of these lignocellulosic matrices triggers the expression of a diverse array of cellulolytic and hemicellulolytic enzymes, as summarized in Table 2.

Table 2. Enzymatic activities (U/mL) produced by *M. thermophilus* (M), *T. hirsuta* (T), and their co-culture (C) using different lignocellulosic substrates.

Culture	Enzymes (U/mL)											
	FPase	EG	CBH	BGL	BXI	XIN	XEG	ARF	ARA	AXE	MAN	LAC
***M. thermophilus* (40 °C)**												
SCB	0.05±0.01	0.09±0.00	0.11±0.02	0.04±0.00	0.11±0.00	2.02±0.22	0.12±0.02	0.03±0.00	0.11±0.01	0.05±0.00	0.06±0.02	0.00±0.00
CC	0.06±0.01	0.10±0.00	0.15±0.02	0.05±0.00	0.15±0.02	1.96±0.14	0.16±0.00	0.04±0.00	0.15±0.02	0.03±0.01	0.08±0.00	0.00±0.00
CS	0.07±0.01	0.10±0.00	0.10±0.01	0.02±0.00	0.15±0.01	6.73±0.54	0.12±0.00	0.01±0.00	0.15±0.01	0.07±0.01	0.08±0.00	0.00±0.00
***T. hirsuta* (30 °C)**												
SCB	0.00±0.00	0.04±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.09±0.01	0.04±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01	7.53±0.65
CC	0.00±0.00	0.06±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.26±0.10	0.03±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01	6.80±0.92
CS	0.00±0.00	0.06±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.60±0.08	0.19±0.06	0.00±0.00	0.00±0.00	0.00±0.00	0.03±0.01	9.99±1.29
Co-culture (M+T) (35 °C)												
SCB	0.07±0.01	0.10±0.01	0.12±0.01	0.29±0.03	0.05±0.00	8.52±0.18	0.30±0.01	0.02±0.00	0.04±0.00	0.01±0.00	0.04±0.00	5.79±0.92
CC	0.08±0.01	0.13±0.01	0.11±0.02	0.30±0.03	0.06±0.02	9.06±0.54	0.43±0.05	0.03±0.01	0.08±0.01	0.03±0.00	0.05±0.01	5.57±0.50
CS	0.09±0.00	0.13±0.00	0.43±0.02	0.68±0.04	0.14±0.01	8.63±0.43	0.46±0.04	0.04±0.00	0.11±0.01	0.04±0.00	0.06±0.01	7.71±0.47

EG = endoglucanase; CBH = cellobiohydrolase; BGL = β-glucosidase; BXI = β-xylosidase; XIN = xylanase; XEG = xyloglucanase; ARF = arabinofuranosidase; ARA = arabinanase; AXE = acetyl xylan esterase; MAN = mannanase; LAC = laccase. Values represent the mean ± SD (n = 3).

The enzymatic profiles revealed that corn straw (CS) was the most effective inducer for the majority of the targeted hydrolases, likely due to its higher hemicellulose accessibility and lower lignin recalcitrance. Specifically, the co-culture system in CS yielded peak activities for several critical components of the cellulolytic complex, including FPase (0.09 ± 0.00 U/mL), endoglucanase (EG, 0.13 ± 0.00 U/mL), cellobiohydrolase (CBH, 0.43 ± 0.02 U/mL), β-glucosidase (BGL, 0.68 ± 0.04 U/mL). This synergy extended to the hemicellulolytic machinery, with maximum titers observed for xylanase (XIN, 8.63 ± 0.43 U/mL), xyloglucanase (XEG, 0.46 ± 0.04 U/mL), and β -xylosidase (BXI, 0.14 ± 0.01 U/mL).

Interestingly, while certain specialized enzymes reached their highest levels in monocultures, such as laccase (LAC) by *T. hirsuta* (9.99 ± 1.29 U/mL) and arabinanase (ARA), acetyl xylan esterase (AXE), and mannanase (MAN) by *M. thermophilus*, the co-culture maintained remarkably high relative production levels. The co-culture system successfully preserved 73% of ARA, 57% of AXE, 75% of MAN, and 67% of LAC activity compared to the highest activity observed in monocultures. This retention is particularly relevant, as it demonstrates that the "thermal midpoint" of 35 °C identified earlier allows for a consolidated bioprocess that yields a more comprehensive enzymatic cocktail in a single fermentation step.

The effectiveness of this microbial consortium stems from the integration of two distinct metabolic machinery: the robust holocellulolytic apparatus of the thermophilic Ascomycete *M. thermophilus* [19,20] and the specialized ligninolytic system of the Basidiomycete *T. hirsuta* [21]. While

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thermophilic fungi are recognized for producing highly stable endoglucanases and xylanases [22–24], the inclusion of *T. hirsuta* ensures the presence of laccases, which are essential for navigating the lignin barriers of LCB. This enzymatic complementarity is crucial for the complete deconstruction of the plant cell wall, providing a more balanced ratio of accessory enzymes that can act synergistically during biomass saccharification, resulting in a more efficient and functionally integrated enzymatic system for lignocellulosic biomass deconstruction

2.3.1. Substrate-Driven Enzymatic Induction and Consortia Synergy

The enzymatic secretome was strongly influenced by the lignocellulosic substrate used as an inducer. As demonstrated in Table 2, corn straw (CS) emerged as the most effective substrate for stimulating the synthesis of the holocellulolytic complex. This enhanced induction is consistent with its higher hemicellulose content and structural accessibility, which provide a broader range of molecular signals for enzyme expression by both *M. thermophilus* and *T. hirsuta*.

2.3.1.1. Cellulolytic and Hemicellulolytic Enhancement in Co-Culture

A key observation was the superior performance of the co-culture compared to individual fermentations for most hydrolytic enzymes. Using CS as substrate, the co-culture yielded peak activities for FPase (0.09 U/mL), EG (0.13 U/mL), CBH (0.43 U/mL), and BGL (0.68 U/mL). This enhancement may be attributed to complementary metabolic pathways and improved substrate deconstruction, facilitating enzyme accessibility and reducing product inhibition. A similar trend was observed for hemicellulolytic enzymes, with xylanase (XIN) reaching 8.63 U/mL in co-culture, representing a significant increase compared to monocultures.

2.3.1.2. Preservation of Accessory and Ligninolytic Activities

While certain specialized enzymes such as arabinanase (ARA), acetyl xylan esterase (AXE), and mannanase (MAN) reached their maximum activities in *M. thermophilus* monocultures, and laccase (LAC) was exclusively produced by *T. hirsuta*, their levels in co-culture remained functionally relevant. The co-culture maintained 73% of ARA, 75% of MAN, and 67% of LAC activity compared to their respective monoculture.

This retention represents a key advantage for integrated biomass deconstruction. Rather than requiring multiple fermentation and downstream processing steps, the co-culture provides a single-step enzymatic system that combines the ligninolytic capacity of *T. hirsuta* with the robust glycosyl hydrolase machinery of *M. thermophilus*. This enzymatic complementarity is essential for overcoming the structural recalcitrance of plant cell walls, as laccases facilitate lignin modification while cellulases and hemicellulases access the polysaccharide core, resulting in a more efficient and integrated saccharification process.

2.4. Biomass Saccharification: The Role of Galactolipase as a Synergistic Accessory Enzyme

The efficiency of enzymatic saccharification is often limited by the structural recalcitrance of plant cell walls, particularly due to the presence of lignin and lipid-rich membrane structures that restrict enzyme accessibility. To evaluate the performance of the developed enzymatic systems, holocellulolytic extracts from the *M. thermophilus* and *T. hirsuta* co-culture were applied to different lignocellulosic substrates. This study specifically evaluated the impact of supplementing the fungal consortium with the *B. lata* BL02 galactolipase (GL) on reducing sugar (RS) release.

As shown in Table 3, the combined enzymatic system (co-culture + GL) consistently outperformed individual fungal extracts across all tested substrates. While the co-culture alone already exhibited improved saccharification compared to the *M. thermophilus* monoculture, due to the ligninolytic contribution of *T. hirsuta*, the addition of GL resulted in a significant increase in sugar release ($p < 0.01$).

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Table 3. Saccharification yields (mg/g of dry biomass) of lignocellulosic and plant-derived substrates after 24 h of enzymatic hydrolysis.

Enzymatic Consortia	Saccharification yield (mg/g of dry biomass)				
	SCB	SP80-3280	CS	SCGL	SL
M. thermophilus	198.3±13.7[a]	137.7±5.0[a]	232.0±25.0[a]	123.3±6.7[a]	170.7±18.7[a]
Co-culture (M+T)	206.0±25.7[a]	149.7±9.0[a]	233.7±21.3[a]	125,3±11.0[a]	186.7±15.0[a]
LIP/GL	12.7±4.3[b]	7.3±4.0[b]	69.0±5.3[b]	33.0±2.0[b]	164.0±7.0[a]
Co-culture + LIP/GL	199.0±20.7[a]	137.3±13.0[a]	236.0±15.7[a]	105.0±14.0[a]	292.0±16.3[b]

Values follow the mean ± SD (n = 3). Different letters in the same column indicate significant differences ($p < 0.01$). Lipase/galactolipase (LIP/GL). SCB: industrial sugarcane bagasse; SP80-3280: variety bagasse; CS: corn straw; SCGL: São Carlos grass leaves; SL: spinach leaves.

2.4.1. The Lipid-Based Structural Barrier and Enzymatic Accessibility

The most striking enhancement in saccharification was observed for spinach leaves (SL), where reducing sugar (RS) release significantly increased from 150.3 mg/g (co-culture) to 235.0 mg/g in the combined consortium, a substantial 56% improvement. Spinach was strategically utilized as a model substrate due to its high concentration of galactolipids (MGDG and DGDG), which are fundamental structural components of chloroplast thylakoid membranes.

These results provide strong evidence for the role of galactolipase as an accessory enzyme. In green biomass, galactolipids form a lipid-based structural barrier that can physically restrict the access of cellulases to the underlying polysaccharide matrix. The *B. lata* BL02 galactolipase exhibits the ability to efficiently hydrolyze these membrane-associated galactolipids, enhancing substrate accessibility and facilitating the action of cellulolytic enzymes.

2.4.2. Variability in Sugarcane Bagasse and Grass Substrates

A significant difference in yields was also noted between industrial SCB (157.0 mg/g) and the SP80-3280 variety (145.9 mg/g). As highlighted by the composition studies of Iryani et al. [25] and Raj & Krishnan [26], sugarcane residues vary inherently based on variety and processing history. The slightly lower yields for SP80-3280 are likely related to the absence of standard industrial milling; conventional milling typically causes extensive physical damage to the cell wall, thereby increasing the surface area and facilitating enzymatic entry, which helps explain the differences observed in saccharification yields.

Sugarcane bagasse is a structurally complex material, typically composed of 35.2-44.9% cellulose, 21.0-35.7% hemicellulose, and 17.8-25.2% lignin. However, intrinsic variability is common; for instance, Kim and Day [27] reported values of 41.6% cellulose and 20.3% lignin, which directly impacts hydrolysis efficiency.

Interestingly, for São Carlos grass leaves (SCGL), the consortium achieved 180.3 mg/g, highlighting the relevance of galactolipase in enhancing saccharification of leaf-derived substrates. While cellulases and hemicellulases are primarily responsible for polysaccharide hydrolysis, these findings demonstrate that integrating ligninolytic (LAC) and galactolipolytic (GL) activities is critical for overcoming structural barriers and maximizing biomass valorization in biorefinery processes.

2.4.3. Mechanistic Insights into Accessory Enzyme Synergy

The enzymatic extracts obtained from the *M. thermophilus* and *T. hirsuta* co-culture consistently resulted in high RS release across all tested substrates (SCB, SP80-3280, CS, and SCGL). This performance is attributed to the efficient hydrolysis of cellulose and hemicellulose fractions, driven by the high activities of EG, XYN, and XEG detected in the co-culture secretome (Table 2). Notably, in traditional lignocellulosic substrates, the fungal-derived machinery plays the dominant role, as evidenced by no significant differences between fungal systems ($p > 0.01$).

Importantly, spinach was the substrate in which the addition of *B. lata* BL02 galactolipase resulted in the most pronounced increase in saccharification efficiency. This effect is associated with

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the high galactolipid content of spinach leaves, where these lipids can represent up to 60% of the total lipids fraction [28]. The enzymatic hydrolysis of these membrane-associated galactolipids enhances substrate accessibility, facilitating the action of cellulolytic enzymes.

These findings highlight the role of galactolipases as key accessory enzymes in overcoming specific biophysical barriers in plant biomass, supporting their integration into next-generation enzymatic cocktails for advanced biorefinery applications.

2.5. Dual Catalytic Profile: Lipase and Galactolipase Activities of B. Lata Bl02

Galactolipids represent the most abundant class of lipids in plant biomass, serving as a major reservoir of fatty acids within the biosphere [29]. These polar lipids, primarily monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are predominantly localized in the thylakoid membranes [30]. Despite their abundance, the efficient recovery of these high-value components from plant tissues remains a technical challenge. In this context, enzymes exhibiting galactolipase activity are essential tools for the selective hydrolysis and valorization of plant-derived fatty acids.

2.5.1. Hydrolytic Performance on Lignocellulosic and Leafy Biomass

The *B. lata* BL02 ester hydrolase was evaluated for its ability to release reducing sugars, likely associated with the hydrolysis of glycolipid head groups. When applied as a standalone biocatalyst on industrial sugarcane bagasse (SCB) and the SP80-3280 variety, only low levels of RS release were observed (12.7 ± 4.3 and 7.3 ± 4.0 mg/g, respectively). This limited performance is consistent with the low glycolipid content (<0.4%) typically found in stalk-derived lignocellulosic residues [31].

In contrast, a progressive and significant increase in hydrolytic activity was observed when the enzyme was applied to leaf-derived biomasses, which are naturally enriched in membrane-associated galactolipids. The enzyme promoted the release of 33.0 ± 2.0 mg/g for São Carlos grass (SCGL), 69.0 ± 5.3 mg/g for corn straw (CS), and a maximum of 164.0 ± 7.0 mg/g for spinach leaves (SL) (Table 3). This trend is consistent with the higher abundance of galactolipids in leaf tissues and supports the role of the enzyme in the deconstruction of membrane-associated glycolipids.

2.5.2. Substrate Specificity and Kinetic Characterization

The enzymatic versatility of *B. lata* BL02 was further investigated using a library of synthetic and natural substrates (Table 4). The results confirm that the biocatalyst exhibits typical lipase behavior [EC 3.1.1.3], efficiently hydrolyzing p-nitrophenyl (pNP) esters with varying acyl chain lengths. The highest activity among synthetic substrates was observed for pNP-palmitate (C16), reaching 3,151.20 ± 111.9 U/mL, indicating a preference for long-chain hydrophobic substrates. This pattern is consistent with interfacial activation mechanisms commonly associated with lipases [7].

A notable feature of the BL02 enzyme is its high activity on complex natural lipids. The hydrolytic activity on poultry oil reached 3,125.00 ± 88.39 U/mL, highlighting its potential for industrial applications involving fats and oils. Additionally, the enzyme exhibited significant activity toward fractionated wheat oil (2,166.67 ± 72.17 U/mL), which is rich in glycolipids such as MGDG and DGDG. The degradation of these galactolipids, confirmed by TLC analysis (Figure 2), supports the classification of this enzyme as a galactolipase [EC 3.1.1.26].

Table 4. Ester hydrolase activity of *Burkholderia lata* BL02 on synthetic and natural substrates.

Synthetic *p*NP-esters Substrate	Ester hydrolase activity (U/mL)
*p*NP-Acetate (C2:0)	457.80 ± 87.7
*p*NP- Butyrate (C4:0)	581.40 ± 35.4
*p*NP-Decanoate (C10:0)	1,661.20 ± 165.0
*p*NP-Laurate (C12:0)	2,416.70 ± 50.3
*p*NP-Myristate (C14:0)	2,138.90 ± 97.7
*p*NP-Palmitate (C16:0)	3,151.20 ± 111.9

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*p*NP-Stearate (C18:0)	2,879.00 ± 65.7
Triolein 98% (C18:1)	2,376.00 ± 64.0
Natural Lipids Substrate	
Chicken oil	3,125.00 ± 88.39
Wheat oil (Lipowheat®)	2,166.67 ± 72.17
São Carlos grass (*Axonopus compressus*)	222.22 ± 27.78
Spinach (*Spinacia oleracea*)	166.67 ± 27.78
Corn straw (CS)	55.57 ± 2.77
Sugarcane bagasse (SCB)	10.32 ± 2.48

*p*NP: *p*-nitrophenyl. Assay conditions: 55 °C, pH 7.0 (50 mM citrate-phosphate buffer). One unit (U) defined as 1 μmol of product released per minute. All experiments were conducted in triplicate, and values are expressed as mean ± SD.

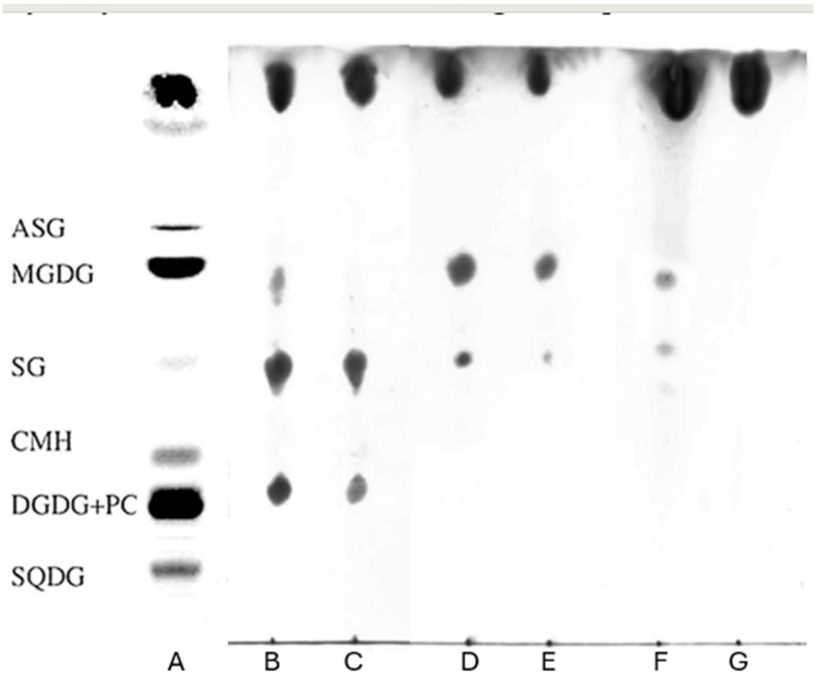

Figure 2. Thin-layer chromatography (TLC) analysis of polar lipids from plant biomass following hydrolysis by the *Burkholderia lata* BL02 galactolipase.

This dual functionality, targeting both neutral triacylglycerols and polar galactolipids, demonstrates the enzyme's substrate versatility and provides a mechanistic basis for the synergistic effects observed during biomass saccharification. By hydrolyzing membrane-associated lipids, the enzyme enhances substrate accessibility, facilitating the action of cellulolytic enzymes in complex lignocellulosic matrices.

2.5.3. Qualitative Analysis of Polar Lipid Hydrolysis via Tlc

The hydrolysis of polar lipids by the *B. lata* BL02 galactolipase was qualitatively evaluated by Thin-Layer Chromatography (TLC). The thylakoid membrane is primarily composed of galactolipids, and their targeted deconstruction is essential for increasing biomass accessibility. Figure 2 illustrates the diverse lipid profiles across the tested biomasses and their respective migration patterns following enzymatic treatment.

In spinach leaves (SL), a substrate recognized for its high chloroplast density, monogalactosyldiacylglycerol (MGDG) was initially present in lower proportions relative to digalactosyldiacylglycerol (DGDG). Following 4 h of incubation with the *B. lata* BL02 enzyme, MGDG was completely hydrolyzed, whereas DGDG was only partially degraded (Figure 2, lanes B and C).

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This preferential hydrolysis of MGDG is consistent with previous reports on both microbial and mammalian galactolipases, which generally exhibit higher catalytic efficiency toward MGDG than DGDG. For instance, studies with *Talaromyces thermophilus* lipase demonstrated significantly higher activity on MGDG, attributed to differences in substrate accessibility and interfacial behavior [14]. Similarly, comparative analyses of microbial lipases and pancreatic enzymes have shown that the presence of a second galactose moiety in DGDG negatively affects enzymatic performance [13].

This effect is primarily associated with the increased size and polarity of the digalactosyl head group, which can impair enzyme adsorption at the lipid–water interface and reduce catalytic efficiency. Experimental and modeling studies suggest that the second galactose introduces steric hindrance and limits the proper accommodation of the substrate within the active site, particularly in enzymes with restricted access channels or partial lid domains [14]. In contrast, the smaller monogalactosyl head group of MGDG facilitates enzyme–substrate interaction, resulting in faster and more complete hydrolysis.

In contrast, the lipid profile of São Carlos grass leaves (SCGL) revealed the absence of detectable DGDG, with MGDG being only partially hydrolyzed within the same timeframe (Figure 2, lanes D and E). Corn straw (CS) exhibited a similar polar lipid distribution, although at lower overall concentrations. In this case, MGDG was fully consumed after 4 h of reaction (Figure 2, lanes F and G). These variations in hydrolysis rates highlight how the lipid architecture of each biomass, particularly the relative abundance of polar lipids and the presence of protective cuticular waxes, directly influences the efficiency of galactolipase activity.

The selective deconstruction of membrane-associated lipids provides a mechanistic explanation for the synergistic effects observed during saccharification. Although galactolipase alone does not generate high levels of reducing sugars, its ability to disrupt lipid barriers within thylakoid membranes represents a critical structural modification. By removing these lipid-based constraints, the enzyme enhances substrate accessibility, facilitating the action of cellulases and hemicellulases on the polysaccharide matrix. This finding reinforces the role of *B. lata* BL02 galactolipase as a strategic accessory enzyme in integrated biorefinery processes aimed at the efficient valorization of green biomass.

Although galactolipid hydrolases are widely distributed in nature, their physiological abundance is typically low, and their isolation from native plant or animal tissues presents significant technical challenges [32–34]. Consequently, the identification of robust microbial sources with high galactolipase activity remains a major objective in industrial biotechnology. In this context, the lipolytic profile of *Burkholderia lata* BL02 toward polar lipids is particularly noteworthy.

Lane A: lipid standards; Lane B: spinach control; Lane C: spinach after 4 h of hydrolysis; Lane D: São Carlos grass control; Lane E: São Carlos grass after 4 h of hydrolysis; Lane F: corn straw control; Lane G: corn straw after 4 h of hydrolysis. Lipid standards: SQDG, sulfoquinovosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PC, phosphatidylcholine; CMH, monohexosylceramide; SG, steryl glycoside; MGDG, monogalactosyldiacylglycerol; ASG, acyl steryl glycoside. Mobile phase: chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v). Visualization: iodine vapor.

As shown in Table 5, the activity of the *B. lata* BL02 enzyme was compared with well-characterized mammalian galactolipases, such as pancreatic lipase-related proteins (PLRP2), as well as several widely used microbial lipases.

The *B. lata* BL02 enzyme exhibits a remarkably high specific activity toward MGDG and DGDG (2,700 U/mg). These values are substantially higher than those reported for representative microbial lipases, such as those from *Fusarium solani* and *Thermomyces lanuginosus*. While most microbial lipases display a strong preference for neutral triacylglycerols with limited activity toward polar lipids, the BL02 enzyme maintains comparable activity toward both neutral and galactolipid substrates.

When compared to mammalian enzymes, which are evolutionarily adapted for complex lipid digestion, the *B. lata* BL02 enzyme exhibits higher activity than human PLRP2 and is only surpassed by guinea pig PLRP2, one of the most active galactolipases described in the literature. This level of

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activity in a bacterial enzyme represents a promising feature, as microbial production via fermentation offers a more scalable and cost-effective alternative to extraction from animal tissues or recombinant mammalian systems.

Table 5. Comparative specific activity (U/mg) of lipases toward neutral triacylglycerol (tributyrin, C4:0) and polar galactolipids (MGDG and DGDG).

Enzyme Source	Tributyrin (C4:0) (U/mg)	Galactolipids (MGDG/DGDG) (U/mg)
B. lata BL02 Lipase	8.345 ± 132	2.700 ± 143
rGPLRP2[a]*	2.700 ± 300	9.795 ± 105
rHPLRP2[b]*	1.250 ± 150	4.762 ± 85
*Fusarium solani** Cutinase	2.596 ± 96	1.284 ± 45
*Thermomyces lanuginosus** Lipase	7.834 ± 850	1.122 ± 51
Candida antarctica A* Lipase	309 ± 11	176 ± 7
*Rhizomucor miehei** Lipase	413 ± 44	126 ± 4
*Candida rugosa** Lipase	753 ± 44	20 ± 1
*Rhizopus oryzae** Lipase	3.375 ± 270	41 ± 3
*Pseudomonas glumae** Lipase	1.179 ± 35	0
*Pseudomonas cepacia** Lipase	86 ± 8	0
*Penicillium camembertii** Lipase	875 ± 10	0
Yarrowia lipolytica LIP2* Lipase	8.102 ± 590	0
Candida antarctica B* Lipase	670 ± 15	0

[a]: Guinea pig pancreatic lipase-related protein; [b]: human pancreatic lipase-related protein. Except for the lipase produced by *B. lata* BL02 in this study, the values for the other enzymes were obtained from Amara et al. [13].

2.5.4. Biotechnological Implications of B. Lata Bl02 Galactolipase for Biorefineries

The ability of *B. lata* BL02 to efficiently hydrolyze both neutral triacylglycerols and polar galactolipids establishes this enzyme as a versatile dual-function biocatalyst. Previous biochemical characterization by our group [17,18,35] consistently supports its potential to broaden the scope of lipid biotransformations.

In the specific context of lignocellulosic biomass valorization, this dual activity provides a mechanistic basis for the enhanced saccharification observed for leaf-derived substrates (Table 3). By targeting membrane-associated galactolipids within thylakoid structures, the enzyme disrupts lipid barriers that limit enzyme accessibility, thereby facilitating the action of cellulolytic and hemicellulolytic systems. This effect is particularly relevant for green biomasses, where galactolipids constitute a significant fraction of the lipid matrix.

Importantly, the integration of *B. lata* BL02 galactolipase into enzymatic consortia represents a strategic approach to improve biomass deconstruction efficiency, enabling more complete utilization of complex feedstocks. These findings highlight the role of important accessory enzymes in overcoming specific structural constraints and support the development of advanced enzymatic systems tailored for next-generation, sustainable, and consolidated biorefinery processes.

2.6. Proteomics and Three-Dimensional Structure Modeling of the Burkholderia Lata Bl02 Galactolipase

The amino acid sequence of the lipase/galactolipase from *Burkholderia lata* BL02 was partially characterized through proteomic analysis. Peptide sequencing via microUPLC-ESI-qTOF-MS yielded fragmentary sequences, enabling a partial reconstruction of the enzyme. Structural prediction was therefore guided by sequence similarity with homologous lipases from closely related Burkholderia species.

The structural characterization of the *B. lata* BL02 enzyme is particularly relevant given its catalytic versatility, including thermal stability, solvent tolerance, and activity toward both neutral and polar lipids. Among the analyzed homologs, the triacylglycerol lipase from *B. lata* ATCC 17760 showed the highest similarity to the identified fragments (155 out of 364 residues), followed by lipases

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from *B. cenocepacia* Bp9145 (154/364) and a cholesterol esterase from *B. cepacia* GG4 (153/364) (Figure 3C).

Protein structure prediction was performed using AlphaFold2, a deep learning-based tool for protein structure prediction [36]. The resulting models were structurally inspected and compared with known α/β-hydrolase folds (Figure 3A,B).

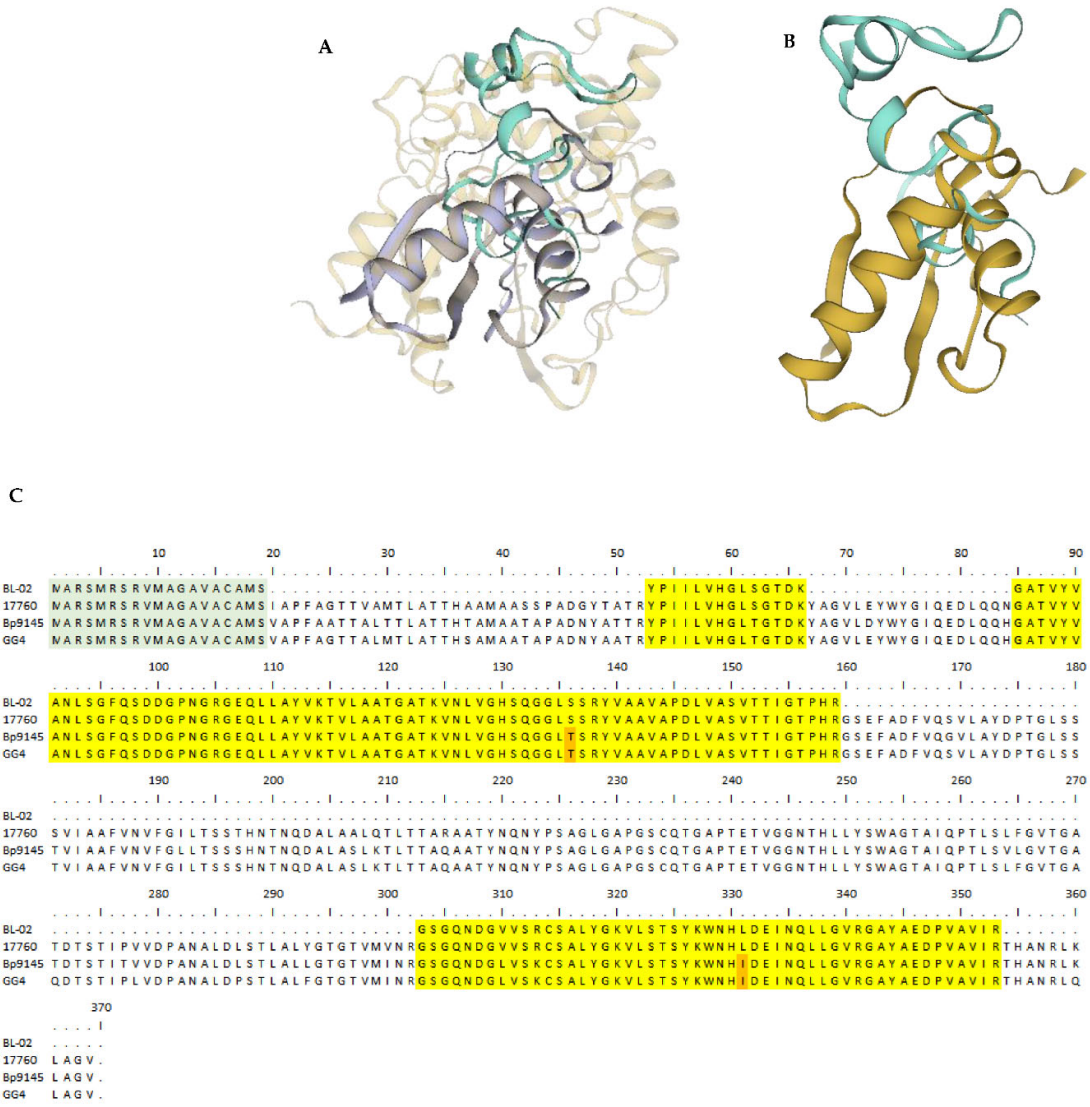

Figure 3. Predicted structural models (A, B) and multiple sequence alignment (C) of the *Burkholderia lata* BL02 lipase compared to reference *Burkholderia* enzymes. (A) Structural superposition of the reference lipase from *B. lata* ATCC 17760 (tan) with the predicted structural model of BL02 (cyan), based on experimentally identified peptide fragments. (B) Predicted three-dimensional structure of the BL02 enzyme. (C) Multiple sequence alignment including *B. lata* ATCC 17760, *B. cenocepacia* Bp9145, and *B. cepacia* GG4. Conserved residues are highlighted, with gaps represented by dashes. Yellow boxes indicate residues identified by LC-MS/MS, and green boxes correspond to predicted signal peptides.

However, the predicted three-dimensional (3D) model should be considered preliminary, as it is based on partial sequence data and inferred homology. Although AlphaFold2 provides high-confidence predictions, the absence of a complete primary sequence limits the accuracy of the structural model. Further experimental approaches, such as full gene sequencing or cloning, will be necessary to establish definitive structure–function relationships.

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The *B. lata* BL02 enzyme has been previously characterized as a galactolipase capable of efficiently hydrolyzing galactolipids [17,18], a substrate class typically resistant to conventional lipases. Based on the current structural insights, the molecular determinants underlying this atypical substrate preference remain to be fully elucidated. Possible structural features, such as differences in the lid domain or a more accessible active site cleft, may facilitate the accommodation of bulky polar head groups, including the galactose moieties of MGDG and DGDG. Elucidating these features may provide valuable insights into the evolution of substrate specificity within the *Burkholderia* genus.

Therefore, further studies integrating structural biology, site-directed mutagenesis, and advanced computational approaches, including molecular dynamics simulations, will be essential to identify the molecular determinants underlying galactolipase activity. Such insights may have significant implications for the development of tailored biocatalysts and for enzyme engineering strategies aimed at expanding substrate scope and improving catalytic performance in high-value industrial applications, particularly within the biorefinery sector.

2.7. Biocatalytic Synthesis of C6 and C5 Sugar Fatty Acid Esters (Sfaes)

The synthetic potential of the lyophilized lipase from *B. lata* BL02 was evaluated through the esterification of carbohydrates with long-chain fatty acids, resulting in the formation of bio-based surfactants with potential applications in the food and cosmetic industries. D-glucose (C6), D-xylose (C5), and D-galactose (C6) were used as acyl acceptors, while oleic acid (C18:1) served as the acyl donor. To overcome the inherent solubility mismatch between polar sugars and hydrophobic fatty acids, the reactions were conducted in different solvent systems, including pure DMSO and binary mixtures of DMSO with isopropanol or butanol (1:3, v/v).

2.7.1. Influence of Solvent System and Carbohydrate Structure

The nature of the reaction medium played a significant role in esterification efficiency. As summarized in Table 6, conversion yields in pure DMSO were limited, reaching a maximum of 15.66 ± 2.81% for xylose after 48 h. In contrast, the use of binary solvent systems substantially enhanced catalytic performance. The highest conversion yields were achieved in DMSO:isopropanol for glucose (54.66 ± 2.79%) and in DMSO:butanol for xylose (55.18 ± 6.75%). These results suggest that the presence of secondary alcohols as co-solvents improves substrate–enzyme interactions and enhances the solubility of reaction intermediates, which is consistent with previous reports on the valorization of lignocellulosic-derived sugars [37].

In contrast, galactose exhibited poor performance as an acyl acceptor, with conversion yields not exceeding 16% under any tested condition. Under identical reaction parameters in DMSO:isopropanol, galactose conversion reached only approximately 3% of that observed for glucose. This behavior can be attributed to its lower solubility in the reaction medium and to the specific spatial orientation of its hydroxyl groups, which may impose steric hindrance within the catalytic pocket of the *B. lata* BL02 enzyme.

Table 6. Conversion yields (%) for the synthesis of sugar esters catalyzed by B. lata BL02 lipase.

Solvent	Sugar	Conversion yield (%)			
		12h	24h	36h	48h
DMSO	Glucose	3.36±0.44	5.33±0.83	11.39±1.62	14.92±1.32
	Xylose	4.74±0.88	7.45±1.36	13.53±1.65	15.66±2.81
	Galactose	1.73±0.35	2.66±0.54	5.29±1.05	8.45±0.91
DMSO:Isopropanol (1:3)	Glucose	12.52±1.02	20.80±1.57	42.56±2.06	54.66±2.79
	Xylose	8.89±0.64	13.53±1.45	28.48±1.83	36.06±1.65
	Galactose	0.88±0.18	2.13±0.28	1.21±0.35	1.66±0.46
DMSO:Butanol (1:3)	Glucose	7.12±0.33	9.58±0.50	22.68±0.98	40.99±3.58
	Xylose	12.47±1.35	18.26±2.08	38.61±3.06	55.18±6.75
	Galactose	1.61±0.47	2.47±0.83	9.82±1.62	15.67±1.54

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The synthetic efficiency of the free, lyophilized *B. lata* BL02 lipase is particularly relevant when compared to established industrial biocatalysts. Zago et al. [38] evaluated several commercial lipases for the synthesis of galactose oleate; while immobilized preparations such as Lipozyme RM IM and Novozym 435 achieved high conversion yields (>70% within 2 h), these systems relied on highly optimized conditions, including the use of ionic liquids. In contrast, non-immobilized lipases, such as those from *Candida rugosa*, typically exhibit reduced stability and activity in polar organic solvents.

Notably, *B. lata* BL02, even in its crude lyophilized form, achieved conversion yields above 55% for glucose and xylose, indicating a high degree of intrinsic robustness and solvent tolerance. Previous studies using Novozym 435 reported yields of 84%, 74%, and 56% for fructose, sucrose, and lactose oleates, respectively, but under longer reaction times (up to 72 h) and often at elevated temperatures [39]. The performance of the BL02 enzyme highlights its potential for the development of cost-effective, non-immobilized biocatalytic systems for sugar fatty acid ester (SFAE) production, particularly when utilizing C5 and C6 sugars derived from lignocellulosic hydrolysates.

3. Materials and Methods

3.1. Microbial Strains and Growth Kinetics

The biocatalysts used in this study were derived from two distinct fungal lineages: the mesophilic white-rot fungus *Trametes hirsuta* GMA-01 [21] and the thermophilic species *Mycothermus thermophilus* (formerly *Scytalidium thermophilum*) CBS 619.91 [40]. The strains were maintained through periodic subculturing on Potato Dextrose Agar (PDA) at 4 °C.

To determine the optimal thermal profile for biomass production and enzyme secretion, mycelial growth kinetics were evaluated at 30, 35, and 40 °C. The fungi were inoculated at the center of Petri dishes containing PDA and incubated in a B.O.D (Biochemical Oxygen Demand) incubator (Tecnal, Brazil). The radial expansion was monitored daily for seven days, measuring the radial growth rate (mm/day) of the colonies. All assays were performed in triplicate.

3.2. Dual Culture (Confrontation) Assays

To assess the compatibility of these strains for potential co-cultivation or synergistic enzyme production, a dual culture (confrontation) assay was performed. The antagonistic potential between *T. hirsuta* and *M. thermophilus* was evaluated by inoculating both strains on opposite poles of a PDA plate, positioned approximately 1.5 cm from the edges.

The plates were incubated at 30, 35, and 40 °C for up to 15 days. Fungal interactions were monitored daily, focusing on radial growth, contact inhibition, overgrowth behavior, and inhibition zone formation (halos). All experiments were performed in triplicate.

3.3. Enzyme Production

3.3.1. Production of Laccase and Holocellulases by Filamentous Fungi Trametes Hirsuta (Gma-01) and Mycothermus Thermophilus (Cbs 619.91)

The production of laccases and holocellulolytic enzymes (cellulases and hemicellulases) by *T. hirsuta* GMA-01 and *M. thermophilus* CBS 619.91 was carried out via submerged fermentation (SmF). Experiments were conducted in 125 mL Erlenmeyer flasks containing 25 mL of Barratt's minimal medium [41], supplemented with 1% (w/v) of specific lignocellulosic biomass (LCB) as the sole carbon source and enzyme inducer. The substrates evaluated included corn cob (CC), corn straw (CS), and sugarcane bagasse (SCB).

For individual cultures, flasks were inoculated with either a 5 mm diameter agar plug of T. hirsuta (incubated at 30 °C) or 1 mL of a spore suspension (10^7 spores/mL) of *M. thermophilus* (incubated at 40 °C). For the co-culture experiments, both inocula were added simultaneously, and the incubation was performed at 35 °C, based on prior growth kinetics analysis. All flasks were agitated at 120 rpm for 120 h, and samples were collected daily for enzymatic assays. The resulting

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crude extracts were recovered by vacuum filtration through Whatman No. 1 filter paper and stored at 4 °C for subsequent assays.

3.3.2. Bacterial Lipase/Galactolipase Production by Burkholderia Lata Bl02

The lipolytic biocatalyst was produced using the *Burkholderia lata* BL02 strain. A pre-inoculum was prepared in 125 mL Erlenmeyer flasks containing 50 mL of Luria-Bertani (LB) broth, incubated at 35 °C and 180 rpm for 48 h. The cell density was adjusted to approximately 10^8 cells/mL based on direct counting using a Neubauer chamber.

Subsequently, 1 mL of this culture was transferred to 250 mL flasks containing 50 mL of fermentation medium. Cultivation was carried out at 35 °C and 180 rpm for 72 h. The production medium was formulated with (g/L): K_2HPO_4 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), NaCl (0.38), $FeSO_4 \cdot 7H_2O$ (0.01) and $(NH_4)_2HPO_4$ with initial pH adjusted to 8.0, as previously described [42].

To induce lipase/galactolipase production, refined poultry oil (Fricock, Rio Claro/SP, Brazil) was added at a concentration of 12.5 mL/L. After fermentation, the culture was centrifuged at 8,500 × g for 20 min at 4 °C. The cell-free supernatant, containing the extracellular enzymatic complex, was collected and used directly for biochemical characterization and biomass hydrolysis.

3.4. Analytical Assays and Enzymatic Quantification

The hydrolytic activities of the holocellulolytic complex (cellulases and hemicellulases) were quantified using two complementary approaches: (i) the measurement of reducing sugars released from natural polysaccharides and (ii) the spectrophotometric monitoring of p-nitrophenol (pNP) liberated from synthetic aryl-glycosides. All enzymatic assays were performed in triplicate, and appropriate blanks were included for each condition.

3.4.1. Determination of Endoglucanase and Glucanase Activities

The activities of endoglucanase (EG), xylanase (XYN), arabinanase (ARA), xyloglucanase (XEG), and mannanase (MAN) were determined by quantifying the concentration of reducing sugars according to the 3,5-dinitrosalicylic acid (DNS) method [43]. The reaction mixtures consisted of 25 µL of a 1% (w/v) specific substrate solution, 10 µL of 50 mM sodium acetate buffer (pH 5.0), and 15 µL of appropriately diluted enzymatic extract to ensure linearity of the reaction. The substrates employed were: carboxymethylcellulose (Sigma-Aldrich®) for EG; beechwood xylan (Sigma-Aldrich®) for XYN; debranched arabinan (Megazyme®) for ARA; tamarind xyloglucan (Megazyme®) for XEG; and locust bean gum (Sigma-Aldrich®) for MAN.

The assays were incubated at 50 °C for 60 min, adapted to micro-scale assay conditions. The reactions were terminated by adding 50 µL of DNS reagent, followed by boiling at 100 °C for 5 min to develop the color. After cooling, the absorbance was measured at 540 nm. Quantification was based on standard curves (0.1–1.0 mg/mL) of glucose, xylose, arabinose, or mannose, as appropriate for each catalytic target. One unit of activity (U) was defined as the amount of enzyme required to release 1 µmol of reducing sugar per minute under the assay conditions.

3.4.2. Determination of Glucosidase Activities

The activities of cellobiohydrolase (CBH), β-D-glucosidase (BGL), β-D-xylosidase (BXI), α-L-arabinofuranosidase (ARF), and acetyl xylan esterase (AXE) were measured using chromogenic p-nitrophenyl substrates [44]. The substrates utilized were p-nitrophenyl-β-D-cellobioside, p-nitrophenyl-β-D-glucopyranoside, p-nitrophenyl-β-D-xylopyranoside, p-nitrophenyl-α-L-arabinofuranoside, and p-nitrophenyl acetate (all from Sigma-Aldrich®).

The reaction mixture, containing 25 µL of 2 mM substrate, 10 µL of 50 mM sodium acetate buffer (pH 5.0), and 15 µL of enzyme, was incubated at 50 °C for 20 min. The process was halted by adding 50 µL of 0.2 M sodium carbonate (Na_2CO_3), which also shifts the pH to alkaline conditions to maximize the yellow color of the liberated p-nitrophenol. The absorbance was recorded at 410 nm

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(ε410 ≈ 18,000 M^{-1} cm^{-1}). One unit of enzymatic activity was defined as the amount of enzyme that produces 1 µmol/min of p-nitrophenol under the specified conditions.

3.4.3. Total Cellulolytic Activity (Fpase)

The total cellulolytic activity, commonly referred to as filter paper activity (FPase), was evaluated to estimate the synergistic action of the endo- and exo-glucanase components of the fungal extracts. The assay was conducted according to the standardized method described by Ghose [45]. Briefly, a 1.0 cm × 6.0 cm (50 ± 5 mg) strip of Whatman No. 1 filter paper was immersed in a reaction mixture containing 1.0 mL of 50 mM sodium acetate buffer (pH 5.0) and 0.5 mL of the enzymatic extract.

The reaction was carried out at 50 °C for 60 min. The process was terminated by the addition of 1.5 mL of DNS reagent, and the tubes were subjected to a 5-min boiling step for color development. After cooling and dilution (if necessary), the absorbance was measured at 540 nm. The concentration of released reducing sugars was determined using a glucose standard curve. One unit of FPase (U) was defined as the amount of enzyme required to liberate 1 µmol of glucose equivalents per minute under the assay conditions.

3.4.4. Laccase Activity

Laccase activity was quantified by monitoring the oxidative coupling of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), as previously described [46]. The reaction system comprised 0,9 mL of 0.4 mM ABTS in 50 mM sodium acetate buffer (pH 5.0) as the substrate, and 100 µL of the enzymatic solution.

The mixture was incubated at 50 °C for 5 min, and the oxidation of ABTS to its cation radical (ABTS+) was monitored spectrophotometrically by the increase in absorbance at 420 nm (ε420 = 36,000 M^{-1} cm^{-1}). One unit of laccase activity (U) was defined as the amount of enzyme necessary to oxidize 1 µmol of ABTS per minute under the specified conditions.

3.4.5. Ester Hydrolase Activity Assays

The catalytic potential of the ester hydrolases produced by *B. lata* BL02, including lipases and galactolipases, was evaluated through two distinct methodologies: (i) hydrolysis of natural galactolipids from plant biomass and (ii) kinetic assays using synthetic p-nitrophenyl esters. All assays were performed in triplicate with appropriate controls, excluding the enzyme.

3.4.5.1. Galactolipase Activity on Natural Substrates

To assess the enzymatic extract's ability to deconstruct plant membrane lipids, three natural substrates were used individually: spinach leaves (*Spinacia oleracea*), São Carlos grass (*Axonopus compressus*), and a fractionated wheat oil rich in glycolipids (Lipowheat®). The plant tissues were initially autoclaved (121 °C, 15 min) to ensure complete inactivation of endogenous enzymes, followed by lyophilization and homogenization to a particle size of 1–2 mm.

The hydrolysis assays were conducted in 3 mL reaction tubes containing 30 mg of the prepared substrate and 0.9 mL of Tris-HCl buffer (50 mM, pH 8.0, containing 150 mM NaCl). After the addition of 1.0 mL of the crude extract (250 U/mL), the mixture was incubated at 50 °C under constant agitation (120 rpm) for 60 min. The reaction was terminated by a lipid extraction step using 3.5 mL of Folch reagent ($CHCl_3$:CH_3OH, 2:1, v/v) [47]. After phase separation via centrifugation (4,000 rpm for 5 min), the organic phase was recovered for the quantification of free fatty acids (FFA) via titrimetry using NaOH (0.05 M). In parallel, the release of galactose equivalents in the aqueous fraction was quantified using the DNS method [43], with absorbance measured at 540 nm against a D-galactose standard curve (0.1–1.0 mg/mL).

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3.4.5.2. Substrate Specificity and Lipase Activity

The substrate preference of the *B. lata* BL02 lipase (LIP) was determined using a series of p-nitrophenyl (pNP) fatty acid esters with varying carbon chain lengths: acetate (C2), butyrate (C4), decanoate (C10), laurate (C12), myristate (C14), palmitate (C16), and stearate (C18), all sourced from Sigma-Aldrich®.

The assays (100 µL of 2 mM substrate, 800 µL of 50 mM citrate-phosphate buffer, pH 7.0, containing 0.45% (w/v) Triton X-100, and 100 µL of enzymatic solution) were incubated at 55 °C for 1 min, and hydrolysis was monitored by absorbance at 410 nm. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol/min of p-nitrophenol.

In addition, the hydrolytic activity toward natural lipid substrates was determined by titrimetric analysis, adapted from Stuer et al. [48]. The assays were performed using spinach leaves (*Spinacia oleracea*), São Carlos grass (*Axonopus compressus*), corn straw, and fractionated wheat oil (Lipowheat®), with triolein and refined poultry oil included as a comparative control.

The reaction system was prepared by emulsifying 200 mg of substrate in 50 mM citrate-phosphate buffer (pH 7.0) containing 6% (w/v) Triton X-100. The reaction was initiated by adding 1.0 mL of enzymatic extract to 5.0 mL of substrate emulsion and incubating at 55 °C under agitation (300 rpm) for 30 min. The reaction was terminated by adding 16 mL of an acetone–ethanol solution (1:1, v/v) to denature the enzyme and stabilize the released free fatty acids (FFAs). The FFAs were quantified by titration with 0.05 M NaOH using phenolphthalein as an indicator. One unit of activity (U) was defined as the amount of enzyme required to release 1 µmol of fatty acid per minute under the assay conditions. Blanks without enzyme were included for all assays.

3.5. Enzymatic Hydrolysis and Saccharification of Lignocellulosic Biomass

3.5.1. Biomass Collection and Standardized Pre-Treatment

To evaluate the hydrolytic efficiency of the developed enzymatic consortia, three distinct lignocellulosic biomasses (LCB) were utilized: (i) industrial sugarcane bagasse (SCB), provided by Pedra Agroindustrial (Serrana, SP, Brazil); (ii) sugarcane bagasse from the specific SP80-3280 variety, supplied by RIDESA (UFSCar, Araras, SP, Brazil); and (iii) corn straw (CS) obtained from local agricultural producers in Ribeirão Preto, SP, Brazil. Notably, the SP80-3280 variety was included as a comparative control because it did not undergo the conventional industrial milling and extraction processes, thereby preserving distinct structural characteristics compared to the standard SCB.

All biomass samples were subjected to a cleaning protocol to remove extractive compounds and residual free sugars. The materials were immersed in 92% (v/v) ethanol for 1 h at room temperature, followed by successive washes with deionized water until no reducing sugars were detected in the supernatant by the DNS method. The cleaned biomasses were dried at 50 °C until constant weight and subsequently ground in an SL-32 knife mill. To ensure particle-size uniformity, the materials were sieved through a 25-mesh screen, yielding a fraction with particle sizes <0.70 mm.

3.5.2. Saccharification Assays

The enzymatic hydrolysis assays were performed in a final liquid volume of 1.1 mL to evaluate the synergistic effect of the fungal holocellulolytic complex (cellulases, hemicellulases) integrated with the auxiliary oxidative enzyme laccases and the bacterial galactolipase (GL). The reaction mixture contained 3% (w/v) of pre-treated LCB, 0.1 mL of 50 mM sodium acetate buffer (pH 5.0), 0.9 mL of the enzymatic extract obtained from the *T. hirsuta* and *M. thermophilus* co-culture, and 0.1 mL of the *B. lata* BL02 galactolipase extract.

Hydrolysis was carried out at 50 °C and 200 rpm for 24 h in a thermomixer (KASVI). Control assays were performed without enzyme and using individual enzymatic systems to evaluate synergistic effects. After incubation, samples were centrifuged at 4,800 × g for 10 min at 4 °C. The

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concentration of reducing sugars, expressed as glucose equivalents, was quantified by the DNS method. Results were expressed as mg of reducing sugars per gram of dry biomass (mg/g).

3.6. Biocatalytic Synthesis of Sugar Fatty Acid Esters (Sfaes)

The lyophilized enzymatic extract from *Burkholderia lata* BL02 was evaluated for its potential to catalyze the synthesis of sugar fatty acid esters (SFAEs). D-glucose, D-galactose, and D-xylose were used as acyl acceptors, representing the main monosaccharides derived from hydrolysis of lignocellulosic biomass. Oleic acid (C18:1) was used as the acyl donor.

The reactions were carried out in a total volume of 5.0 mL containing 300 mM of sugar and 300 mM of oleic acid (molar ratio 1:1), in the presence of 50 mg of lyophilized enzyme. The reactions were performed in three solvent systems: anhydrous DMSO and DMSO/isopropanol or DMSO/butanol mixtures (1:3, v/v). All systems were prepared under anhydrous conditions.

The reactions were conducted at 50 °C under agitation at 300 rpm for up to 48 h. Samples were collected at defined intervals for analysis. The biocatalytic efficiency was expressed as the conversion yield (%), calculated as the molar ratio of the synthesized sugar ester to the initial carbohydrate concentration. All synthesis experiments were performed in triplicate to ensure reproducibility, and the results were analyzed to determine the influence of the acyl donor chain length on the enzymatic performance.

3.7. Proteomic Investigation of Galactolipase and Structural Modeling

3.7.1. Mass Spectrometry-Based Proteomics (Lc-Ms/ms)

To confirm the identity of the target ester hydrolase, the purified enzyme was first resolved by SDS-PAGE. The protein band corresponding to the lipolytic activity was excised from the gel and subjected to in-gel tryptic digestion. The resulting peptide fragments were analyzed using a micro-link Liquid Chromatography-Electrospray Ionization-quadrupole Time-of-Flight Mass Spectrometry (microUPLC-ESI-qTOF-MS) system at the LaCTAD Proteomics Laboratory (UNICAMP, Campinas, SP, Brazil).

The MS/MS spectra were processed and searched against the UniProt and NCBI databases using specialized algorithms to identify the protein. Identification was considered positive based on the matching of multiple peptides with high confidence scores, specifically targeting the proteome of *Burkholderia lata*.

3.7.2. Proteomic Data Processing and Protein Identification

The raw mass spectrometry data were processed using the ProteinLynx Global SERVER (PLGS) version 3.0.3 (Waters Corporation). Protein identification was performed by searching against a specific *Burkholderia lata* database retrieved from UniProt (Swiss-Prot/TrEMBL). The processing parameters were configured in automatic mode to achieve MS-TOF resolution and determine chromatographic peak width.

Mass tolerances for precursor peptides and fragment ions were dynamically assigned by the software during the evaluation query. The search criteria included at most one missed cleavage site for trypsin. Low and high energy detection thresholds were software-optimized to ensure the capture of high-quality spectra. Proteins were identified based on at least two unique peptides with a confidence level above 95%. For absolute quantification, rabbit Phosphorylase B (UniProt P00489) was utilized as an internal standard, spiked at a concentration of 200 fmol. Protein identification was validated using a false discovery rate (FDR) below 1%.

3.7.3. Molecular Modeling and Structural Prediction

The three-dimensional (3D) structure of the *B. lata* BL02 ester hydrolase was predicted through a hybrid computational workflow. Primary structural templates were generated using AlphaFold2,

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leveraging its deep learning-based folding algorithms to predict high-confidence polypeptide architectures. These models were further refined using MODELLER to ensure proper stereochemical constraints and alignment accuracy [36].

The global and local quality of the resulting tertiary structures was assessed using the QMEANDisCo scoring function [49]. This tool provided a distance-based quality estimation by comparing the model with experimental structures of homologous proteins. Special attention was given to the architecture of the catalytic triad (Ser-Asp-His) and the presence of the "lid" domain, a flexible helical loop characteristic of "true" lipases. This structural analysis was used to support and distinguish the *B. lata* BL02 enzyme from typical lipases and to rationalize its high catalytic affinity toward polar galactolipids.

3.8. Analytical Methods

3.8.1. Lipid Profile Characterization by Thin-Layer Chromatography (Tlc)

The qualitative analysis of the hydrolysis products was performed using Thin-Layer Chromatography (TLC) to monitor the breakdown of complex lipid classes. Following the enzymatic assays, the lipophilic components were recovered by solvent extraction with hexane.

Aliquots (5.0 µL) of the organic phase were applied onto silica gel 60 F254 plates (Merck). The separation of lipid classes, particularly galactolipids (MGDG and DGDG) and neutral lipids, was achieved using a complex mobile phase consisting of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v/v/v/v) [50]. After the chromatographic run, the plates were dried, and the spots were visualized by exposure to iodine vapor. This method allowed the identification, by comparison with standards, of the primary metabolites resulting from the galactolipase-driven deconstruction of plant-derived lipids.

3.8.2. Protein Quantification

Protein quantification during the cultures was performed according to the dye-binding method described by Bradford (1976)with protein concentration calculated from a calibration curve using bovine serum albumin as the standard.

3.9. Statistical Analysis and Experimental Reliability

All experiments were performed in triplicate (n = 3), and results are expressed as mean ± standard deviation.

The assumptions of normality and homoscedasticity were verified using the Shapiro-Wilk and Levene's tests, respectively. Statistical significance across different growth conditions, enzymatic activities, and biomass saccharification yields was assessed via one-way analysis of variance (ANOVA). For specific comparisons between experimental groups, a student's t-test was employed where applicable. All statistical processing and high-resolution graphical representations were performed using Origin Pro 10.0 software (OriginLab Corp., Northampton, MA, USA). A p-value of less than 0.05 ($p < 0.05$) was considered the threshold for statistical significance.

4. Conclusions

This study demonstrates the synergistic performance of an enzymatic consortium for both lignocellulosic biomass deconstruction and the synthesis of value-added bio-based surfactants. The integrated production of laccases and holocellulases by *Trametes hirsuta* GMA-01 and *Mycothermus thermophilus* CBS 619.91, combined with the galactolipase activity of *Burkholderia lata* BL02, was successfully achieved using agro-industrial substrates, supporting the feasibility of a cost-effective and sustainable bioprocess.

Proteomic analysis and structural modeling identified the *B. lata* BL02 enzyme as a versatile ester hydrolase closely related to *Burkholderia* lipases. Although the structural model remains preliminary

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due to partial sequence coverage, it provides useful insights into potential features associated with substrate accommodation, including an accessible catalytic region compatible with bulky polar head groups.

Saccharification assays showed that the incorporation of galactolipase activity enhances the hydrolysis of leaf-derived biomasses. The combined consortium achieved higher reducing sugar yields compared to fungal systems alone, reaching up to 292.0 mg/g for spinach leaves and 236.0 mg/g for corn straw. These results support the role of galactolipase as an important accessory enzyme that facilitates biomass accessibility by targeting membrane-associated lipid components.

In addition, the *B. lata* BL02 enzyme demonstrated relevant catalytic performance in the synthesis of sugar fatty acid esters, achieving conversion yields above 50% for glucose and xylose in binary solvent systems. This behavior indicates good tolerance to organic media and applicability across different carbohydrate substrates derived from lignocellulosic hydrolysates.

Overall, the dual catalytic profile of *B. lata* BL02, combining hydrolytic and synthetic activities, highlights its potential for integration into biorefinery platforms. Further studies focusing on complete sequence characterization, structural validation, and enzyme engineering will be important to better understand its catalytic properties and to expand its applicability in sustainable biotechnological processes.

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Abbreviations

The following abbreviations are used in this manuscript:

U	Unit
TLC	Thin-Layer Chromatography
SmF	Submerged fermentation
pNP	p-nitrophenol
LCB	Lignocellulosic biomass
SFAEs	Sugar fatty acid esters
MGDG	Monogalactosyldiacylglycerol
DGDG	Digalactosyldiacylglycerol
GL	Galactolipase
LIP	Lipase
RS	Reducing sugars
EG	Endoglucanase
CBH	Cellobiohydrolase
BGL	β-glucosidase
XYN	Xylanase
XEG	Xyloglucanase

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BXI	β-xylosidase
ARA	Arabinanase
AXE	Acetyl xylan esterase
MAN	Mannanase
LAC	Laccase
FFA	Free fatty acids

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